# MICROBIOLOGY

**Project title: Survey of Yellowstone Hot Springs for Green Sulfur Bacteria**

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Objective: 1) To survey selected hot springs in Yellowstone National Park for the presence of green sulfur bacteria (GSB); 2) To further characterize and possibly isolate organisms whose 16S ribosomal gene sequences indicate that they may be deeply branching relatives of green sulfur bacteria from selected Yellowstone hot springs. We sought to apply powerful molecular techniques to determine whether we could detect the presence of Chlorobium or other green sulfur bacteria in hot springs in the Mammoth region or in other Yellowstone hot springs that might provide a suitable habitat.

Findings: We designed primers specific for an 800 base pair region of the 16S ribosomal gene and used PCR amplification to screen for the presence of green sulfur bacteria in DNA extracted from 18 different locations in 11 different hot springs. Seven of these hot springs were in the Mammoth Hot Springs region. We also tested Octopus Spring and Mushroom Spring, because the Ward lab has previously detected 16S rRNA genes (E-type) that are closely related to those of green sulfur bacteria (GSB) in these sites. Finally, we tested several locations in a large unnamed pool East of Artist Paint Pots and in effluent from an unnamed hot spring in the Mud Volcano region. These were selected because they had the proper temperature and pH for previously identified thermophilic GSB even though the sulfide content was low. We detected GSB 16S rRNA sequences only in the hot spring from the region East of Artist's Paint Pots.

We subsequently enriched green sulfur bacteria from two different locations in this pool. Two enrichments grow at 36.5C, but a third grows well at 44C. Spectrophotometric analysis of the photosynthetic pigments and PCR analysis with GSB specific primers both confirm that each of these enrichments contains green sulfur bacteria. We have not yet obtained isolates from these enrichments but plan to do this. We also obtained GSB enrichments from two different locations in the Mud Volcano region, one at 36.5C, and one at 44C. Again, the identification was confirmed by both spectrophotometric analysis of the pigments and by amplification with GSB-specific primers, even though we were not successful in detecting the GSB sequences in DNA extracted directly from the hot springs without prior enrichment.

We are working to improve the sensitivity of our PCR analysis since we have still not succeeded in detecting the presence of GSB organisms in three of the four sites from which we subsequently obtained positive GSB enrichments. Obviously the organisms are present, but our assay is not sufficiently sensitive to detect them. In addition, since our enrichments were more successful than our molecular screening, we

intend to collect fresh samples from the Mammoth Hot Springs region and attempt GSB enrichments on these samples.

We have cloned an 800 bp region of the GSB 16S rRNA from each of these enrichments as well as from the DNA of the single site in which we were able to detect GSB sequences directly. Mary Bateson sequenced 40 some clones from the latter site. Comparison with previously sequenced 16S rRNAs using the BLAST tool of GenBank confirms that these are indeed green sulfur bacteria, but that their sequences are not identical to those of any of the organisms in the data base. We will be continuing our sequencing and analysis of these genes.

**Project title: Bacteria Living at Low pH and High Temperature**

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Objective: The main objective is to examine high temperature and low pH springs to find new (i.e. previously undescribed) hyperthermophiles and develop methods to isolate and culture them. There are three specific objectives: 1) examine and quantify existing microbes in selected springs for site reference purposes (phase contrast and epifluorescence light microscopy); 2) analyze and assess low pH high temperature habitats for the presence of microbes defined by their structural features in the electron microscope using scanning and/or transmission electron microscopy; 3) study the growth and growth rates of species in nature by observation of organisms attached to immersed slides. Physical identification of the physiological state and numbers of organisms present at a given site will allow us to design media and establish conditions used for the isolation of microbial cultures.

Findings: A previous examination of sampling sites for microbes provided the result that DNA was present in small objects regarded to be microbes. There were various shapes and arrangements of these seen by light microscopy. At Amphitheater Springs, rod-shaped organisms were present at all temperatures examined but the numbers increased at lower temperatures. Round Sulfolobus-like organisms predominated at Moose Pool (Mud Volcano area) and Great Sulfur Spring (Hayden Valley-Crater Hill area). In media prepared for isolation of organisms in aerobic conditions we found growth at lower temperatures (55 C) in Great Sulfur Spring samples. We examined several sites by electron microscopy and found a diverse collection of organisms from several sites including Frying Pan Spring, Amphitheater Spring, and Sulfur Caldron. Various sizes and arrangements of rods and spheres were the forms seen. These organisms will be isolated in prepared laboratory media.

**Project title: Transition Between Lithoautotrophy and Chemoheterotrophy in *Sulfolobus* Species**

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Objective: To determine the factors which regulate the metabolic status of hyperthermophilic archaea and bacteria in situ. To investigate methods for recovery of viable cells.

Findings: The effect of sample pH, sample concentration, and sample ultrafiltration was examined on the recovery of viable cells from geothermal sites at various locations in the park.

**Project title: Molecular Ecology of Photosynthetic Hot Spring Bacteria that Resemble *Heliothrix Oregonensis***

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Objective: The purpose of this ongoing project is to locate, describe, and compare microbial mat communities in Yellowstone National Park that contain deep red-colored layers dominated by novel red filamentous bacteria. Previous studies, both in our lab and in the labs of collaborators, have demonstrated that red layer bacteria in one Yellowstone community are photosynthetic and use unique molecules to harvest light for energy. Taken together, these data strongly suggest that red layer bacteria are related to, but distinct from, orange filamentous bacteria *(Heliothrix oregonensis)* found in some Oregon hot springs. *Heliothrix* represents an unusual member of the Green Non-Sulfur (GNS) lineage of bacteria, a lineage believed to represent one of the most ancient groups of life on earth. The goal of our work is to use molecular methods (e.g. DNA sequence analysis) to a) better address the identity of the red layer bacteria within each separate community; (b) to compare GNS sequences from different red layer communities to better understand variation, selection, and transfer/origin of these related bacteria; and (c) to improve our understanding of diversity in this evolutionarily important lineage of bacteria.

Findings: To date, we have located approximately twelve distinct red layer communities in Yellowstone in terms of one or more of the following categories we have measured: temperature, pH, photosynthetic pigment properties, or geographical location. In the past year, we specifically surveyed the Shoshone Thermal Basin, adding one new red layer community sample to our target study. Our six-member party backpacked

in. We also surveyed the following thermal areas, all on foot: Potts (escorted, no samples removed), Sentinel Meadows (one sample removed from Sentinel Pool, beyond Mound Spring), and White Creek (no samples removed). We re-sampled four key red layer sites to track potential variation over time: Hillside Springs, Fairy Springs, Spray Geyser, and Imperial Geyser. Previous sampling at Imperial was performed at the main pool. This year, we surveyed and sampled downstream run-off as no red layer was visible in the main pool (likely because the temperature and thermal activity had noticeably increased). We described similar increases at Spray Geyser where last year's flourishing mat had also diminished appreciably.

We are currently using DNA sequencing methods to describe and compare 16S rRNA gene sequences from red layer sites. In 2000, we specifically completed DNA analyses on two key sites (Hillside and Witch Pond), observing, as hypothesized, large numbers of novel GNS 16S rRNA sequences. While our approach is designed to preferentially target red filamentous bacteria using physical separation methods and specific DNA probes, we have also isolated and described many non-red bacterial sequences, each of which is novel. All sequences have been or will be submitted to GenBank. It should be noted that much of the Hillside analysis was completed by eight undergraduates in the context of a research-driven course I teach in molecular biology.

In September, I began three years of NSF funding (Microbial Observatory/Research at Undergraduate Institute Grant) and hired a full-time technician to work on this project. In addition to continuing DNA analyses, we have constructed an extensive public website and database about this work (www.wou.edu/las/natsci_math/biology/boomer/ALLRESEARCH/rlmocover.html). Databased information includes the following: site information over time, pigment analysis, microscopy, and DNA sequence information (including GenBank Accession information, BLAST homology searches, and phylogenetic trees). This grant also supports education, specifically funding research-driven coursework and educational outreach directed at the public schools, all based on analyzing materials from this project (see website above).

**Project title: Recognizing the Signature of Hyperthermophilic Biofilms: Geyserites, Epithermal Deposits and Ancient Cherts**

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Objective: Identify the processes by which hyperthermophilic biosignatures are formed and preserved in the geologic record. Establish framework within which the paleobiology, paleoecology, and paleoenvironments of hyperthermophilic communities can be recognized.

Findings: Determined through a combined microscopy and phylogenetic analysis of hyperthermophilic biofilms that the distribution of distinct hyperhtermophilic communities could not necessarily be correlated with the distribution of specific morphotypes of sinter. Began testing working hypothesis that character-

istics of microbial biofilms are related to specific microenvironments within thermal springs and geysers.

**Project title: Research Experience for Undergraduates: Yellowstone National Park Field Trip, Summer 2001**

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Additional investigator(s): Darla Goeres, Melissa Cahoon, John Neuman

Objective: The Research Experience for Undergraduates program at the Center for Biofilm Engineering, Montana State University recruits talented students in various science, math and engineering disciplines to spend 10 weeks in Bozeman conducting biofilm research, learning effective technical communication skills and debating ethical issues that arise in technical fields of work and study. Yellowstone National Park serves as the perfect location to debate the ethics of harvesting microorganisms from natural environments. The students spent two days in the park observing wild type biofilms and discussing current biofilm research being conducted in the park.

Findings: The trip to Yellowstone Park increased the students' appreciation for field research. Viewing biofilm in a natural environment demonstrated the complex ecology associated with a living biofilm better than any bench-top laboratory system. The students left Yellowstone with a better understanding of the issues surrounding research in a national park.

**Project title: Long-Term Effects of UV Radiation on Biodiversity and Photosynthetic Competence of Hot Spring Bacteria**

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Additional investigator(s): Tracy Norris, Erich Fleming

Objective: To determine if 1-3 month UV exclusion resulted in a change in the species composition of microbial mats in hot springs and whether photosynthetic competence of the cyanobacteria was affected.

Findings: The objective of this study was to determine whether the long-term exclusion of UVR in hot spring microbial mats resulted in an alteration in microbial community composition, such as a population shift to more UV-sensitive species. Over a 1–3 month period microbial mats in three alkaline geothermal streams in Yellowstone National Park were covered with filters that excluded or transmitted UV radiation (UVR). Over some, 25 percent transmission neutral density screens were also used. For mats in the 40–45 C range there were no significant changes in species composition during the summer with or without high or low UV radiation, as assayed by DGGE after PCR amplification of 16S-rRNA genes using general Bacterial and cyanobacterial primers. Although the composition of these microbial communities appeared to be stable, surface layers of cyanobacteria protected from UV radiation (high or low) were not as competent photosynthetically as those maintained with UVR. This decrease was expressed as a loss of the ability to perform at a maximum rate under UVR + visible irradiance. However, even UV-acclimated cyanobacteria performed better when UVR was excluded. It is probable that the large differences observed reflect changes at the level of gene expression rather than changes in species composition.

**Project title: Research Experience for Undergraduates: Day Trip to Yellowstone National Park**

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Additional investigator(s): Dr. Warren Jones, Darla Goeres

Objective: The Research Experience for Undergraduates program at the Center for Biofilm Engineering, Montana State University recruits talented students in various science, math and engineering disciplines to spend ten weeks in Bozeman conducting biofilm research, learning effective technical communication skills and debating ethical issues that arise in technical fields of work and study. Yellowstone National Park serves as the perfect location to debate the ethics of harvesting microorganisms from natural environments. The students spent two days in the park observing wild type biofilms and discussing current biofilm research being conducted in the park.

Findings: The trip to Yellowstone Park increased the students' appreciation for field research. Viewing biofilm in a natural environment demonstrated the complex ecology associated with a living biofilm better than any bench-top laboratory system. The students left Yellowstone with a better understanding of the issues surrounding research in a national park.

**Project title: Ectomycorrhizae of Thermal Soils**

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Objective: To determine effects of thermal alteration of soils on ectomycorrhizae (EM) of lodgepole pine.

Findings: EM communities of pine in thermal soils are significantly different from those in non-altered soils. EM Fungi in thermal soils are not unique to these soils, but are minor players in undisturbed systems, and selected for by thermal alteration.

**Project title: Isolation and Characterization of Microorganisms Extremely Resistant to DNA Damage**

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Objective: Very little is known about strategies adopted by extremophiles to maintain the integrity of their genetic material in very hot environments. To survive, all cells must continuously and accurately repair lesions to their DNA caused by environmental stress. For hyperthermophiles, additional damages are inflicted on their DNA by exposure to elevated temperature. In preliminary studies, we have found that the hyperthermophile Pyrococcus furiosus is extremely resistant to ionizing radiation. Based on these observations we propose the following hypothesis: "Hyperthermophiles resistance to ionizing radiation is due to their unusual ability to repair extensive heat induced DNA double-strand breaks which occur at a much higher rate at elevated temperature. Therefore, hot environments should also be a prime source of highly radioresistant microorganisms."

The proposed project addresses the following questions: 1) What are the highest temperatures under which genome integrity can be maintained? 2) How do hyperthermophiles protect and repair their DNA? To address these questions, we will isolate and characterize novel and highly resistant thermophiles from hot springs in Yellowstone National Park containing elevated radon levels, and exposed to high fluxes of solar radiation. In addition, we will use extreme UV- and g-irradiation as selective pressure during enrichment to eliminate competing radiation sensitive microorganisms. We will investigate the accumulation of DNA lesions from exposure to sublethal doses of radiation and the kinetics of removal of those lesions with the most radiation-resistant isolates. We will assess the performance of the new isolates under simulated space conditions at NASA Goddard Space Flight Center and at the National Institute of Standard and

Technology Synchrotron facility. Long-duration tests will determine the performance limits of the isolates exhibiting the greatest survival potential. In addition, survival and recovery of microbial isolates will be measured after their exposure to full spectrum solar radiation during a Solar Extreme Ultraviolet Rocket Telescope and Spectrograph (SERTS) flight.

Findings: 1) Characterization of thermophilic microorganisms resistant to desiccation, hard vacuum and gamma irradiation: Fourteen strains of thermophiles with optimum temperature from 65 to 75°C were isolated from samples collected in Yellowstone National Park and the Kamchatka Peninsula in Russia. Strains were isolated following exposure of the samples to hard vacuum or gamma irradiation, as selective pressures. The isolates were characterized for their survival to gamma irradiation at doses up to 5,500 Gy. Their exposure to hard vacuum is in progress. We found that all the strains were highly resistant to ionizing radiation with D37 (dose for 37 percent survival) above 3000 Gy. In comparison, the D37 for Escherichia coli is lower than 100 Gy. In addition, the strains isolated with gamma rays as selective pressure showed an increase in survival compared to the strains isolated with hard vacuum. The 14 strains are being characterized at the molecular level using RFLP and 16S rRNA.

2) Exposure of microorganisms to space vacuum and EUV during a SERTS flight. In collaboration with scientists at GSFC, we determined the survival of thermophilic microorganisms to space conditions and EUV radiation (35 to 60 nm) during a SERTS flight. Cell-holders, mirrors, aluminum filters, temperature probes and electronic components were specifically designed and built for this experiment. The two microorganisms, *Deinococcus radiodurans* and the gram negative bacterium PD3D (isolated from Yellowstone National Park, optimum growth temperature 70°C) were selected for their resistance to desiccation and hard vacuum. Cultures were filtered on polycarbonate filters to obtain about 10 e8 cells per filters (single layer of cells). Two filters were mounted on each cell holder, with one of the filters kept in the dark at all times (control for non-EUV exposure). Because of the limited amount of space on the head of the SERTS telescope, only 2 cells holders were used. Aluminum filters placed in front of the samples blocked all UV radiation except for EUVs. The SERTS telescope was successfully launched on a Terrier-Black Brant rocket on July 26, 2000 from the White Sands Missile Range (New Mexico). The rocket reached an altitude of 304 km in 283.5 seconds. The shutters stay open for 441 seconds exposing the microorganisms to space vacuum and EUV radiation. The rocket payload was recovered the same day and the microorganisms were brought back to the laboratory immediately. Following their exposure to space environment, we determined the level of survival of *D. radiodurans* and PS3D cells using cell counts. For both microorganisms, desiccation has little effect on the cell survival compared to the non-desiccated control. Exposure to space vacuum (~10 e-6 Pa) however, decreased cell survival by two and four logs for PS3D and *D. radiodurans* respectively. The most interesting result of this experiment is that exposure to EUV radiation decreased the survival of both organisms by 1 log. This is the first measurement of the effect of EUV on cell survival. We are confirming these results with synchrotron studies. We will identify the DNA lesions resulting from exposure to EUV radiation and investigate the DNA repair mechanisms involved in the repair of these lesions.

**Project title: The Search for Thermophilic *Protozoa* in Yellowstone National Park**

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Objective: The goal of this research is to identify thermophilic *Protozoa* in Yellowstone National Park. Research on thermophilic organisms has so far focused largely on prokaryotes. In the hot springs of Yellowstone, these efforts have been successful, discovering many novel thermophilic organisms and their gene signatures of prokaryotic nature. This suggests that thermophily may not be a highly specialized adaptation of a few obscure microbes but a widespread phenomenon not limited to the *Bacteria* and *Archaea*, but perhaps extending to the eukaryotic domain as well. The possibility that there might exist thermophilic eukaryotes, specifically protozoa, is the focus of this proposal. Needless to say, Yellowstone National Park is one of the very few environments on this planet that can be expected to harbor thermophilic *Protozoa*.

Findings: The samples were collected and transported to our home lab. We extracted DNA from several samples and are working on optimization of the PCR-aided 18ssRNA gene amplification protocol to amplify the target genes.

**Project title: A Survey of *Pilobolus* from Yellowstone National Park**

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Additional investigator(s): Dr. Donald Ruch

Objective: 1) To obtain isolates of *Pilobolus* to examine for differences in DNA sequences and cellular short chain fatty acid composition; 2) To compare isolates from various locations by contrasting morphological characters to DNA sequences and short chain fatty acids; 3) To analyze and compare nucleic acid sequences in the various isolates to compare and contrast taxa; 4) To analyze and compare cellular short chain fatty acids in the various isolates to compare and contrast taxa; 5) To study the characteristics that can be used to identify isolates.

Findings: During 2000, isolates of *Pilobolus* were collected in Yellowstone National Park during July and August. These isolates were collected from mule deer, buffalo, pronghorn, and elk from areas near Buffalo Ford, Indian Camp, Duck Lake and Mammoth Hot Springs. All isolates have been maintained in the laboratory at Indiana University East are being used as part of larger studies to distinguish among the species of *Pilobolus*. Collections of *Pilobolus* from this project are maintained at Indiana University East. It should be noted that isolates of *Pilobolus* do not survive well under cultivation. Most isolates of Pilobolus collected in earlier years have died.

**Project title: Determination of In Situ Growth Rates of Filamentous Sulfur-Oxidizing Bacteria in a Hydrothermal Vent Field in Yellowstone Lake**

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Objective: We will determine the specific growth rate of cells replicating within filaments of *Thiothrix*-like, sulfur-oxidizing bacteria that have attached to metal coupon surfaces positioned in a hydrogen sulfide gradient above a hydrothermal vent. Using published conversion factors, we will describe the chemosynthetic or mixotrophic biomass production of filamentous sulfur-oxidizing bacteria attached to coupons at different hydrogen sulfide concentrations and temperatures.

Findings: During August, 2000, Mary Bay and adjacent areas of Yellowstone Lake were surveyed by SCUBA for hydrothermal vents emitting hydrogen sulfide gas. Three vent fields were located at water depths ranging from 8 to 10 meters that emitted vent fluid with sulfide concentrations ranging from 0.012 to 0.469 mg/L. A vent located at the east end of Mary Bay at a water depth of 8 meters was selected for bacterial colonization and growth studies. Stainless steel coupons were suspended from tripods positioned by SCUBA in the fluid emitted from the vent. Control coupons were suspended above the lake bottom by a tripod positioned 3 meters from the vent in an area where no hydrogen sulfide was detected. At daily intervals over an 87 hour period, coupons were retrieved by SCUBA from both sites, placed in a screw cap test tube filled with surrounding water, brought to the surface, and preserved with 0.5 percent glutaraldehyde. Upon return to the Montana State University Laboratory, the bacteria that had attached to each coupon were stained with a fluorochrome for microscopic visualization. The number of individual cells in the largest filament attached to each coupon was determined.

The motivation for evaluating the number of cells in the largest filament on each coupon is based on our assumption that this filament arose from a single cell that had been one of the early colonizers of the coupon upon exposure to the hydrogen sulfide-containing vent environment. The hydrogen sulfide is thought to serve as the primary energy source for replication of the bacteria attached to the coupon. The attached bacteria replicate inside a filament that extends perpendicular to the coupon surface. The filament elongates as the cells within the filament replicate. By determining how many cells exist within the longest filament attached to coupons recovered at daily intervals, in situ growth rates or generation times can be estimated.

To date we have identified the largest filament on one coupon retrieved after 14 hours exposure to the vent fluid and one coupon exposed for the same period of time to ambient water outside the vent field. A 203 um-long filament containing 66 bacterial cells was the largest filament on the coupon exposed to the vent fluid at a temperature of 52-54C. This equates to a specific in situ growth rate of 0.43 generations/h or a generation time of 2.3 hours over the first 14 hours of exposure. An 82 um-long filament containing 36 cells was the largest filament attached to the coupon exposed to ambient lake water at a temperature of 16C. This latter result indicates that either hydrogen sulfide is diffusing from the lake bed sediment at concentrations below the detection limits of our assay, but at concentrations sufficient to support replication of the observe filamentous bacteria in areas where vents are not apparent, or that these filamentous bacteria can replicate on an available energy source other than hydrogen sulfide.

Between March 1 and July 1, 2001, we will complete the analysis of bacterial growth rates on coupons collected at later times during the 87 hour study. We would like to deploy new coupons in the same area and conduct similar studies during the summer of 2001 in order to evaluate the reproducibility of our Year 1 data. We also want to determine whether the vent is still actively emitting hydrogen sulfide at similar concentrations as those measured during August of 2000.

**Project title: Protein Comparison of Thermophiles and Oral Bacteria**

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Objective: Oral bacterial microflora are extremely diverse (more than 300 different species in the normal oral cavity) and have to survive relatively large temperature and nutritional variations. Thermophilic microorganisms have been fairly well described, but no comparison has been reported with oral bacteria. It is proposed here to compare protein antigens between thermophilic and oral bacteria by immunological and electrophoretic (protein size) techniques. SDS-PAGE electrophoresis will be used to compare the sizes of proteins between representative thermophiles and laboratory strains of oral streptococci (primarily *Streptococcus mutans,* the causative agent of human dental caries). Immunological assays such as ELISA and western blots will be used to compare reactivity between antibodies to protein antigens on *S. mutans* and the thermophiles. It is anticipated that similar proteins will be observed between thermophiles and oral bacteria implying a possible common ancestry.

Findings: Bacterial colonies were isolated on both selective and non-selective petri plates. Selected colonies were propagated and stored frozen until assayed. Samples are currently being collected from human volunteers to compare to park samples. It is anticipated that more park samples will be required due to poor growth of several bacterial colony types upon secondary propagation.

**Project title: Analysis of a Eukaryotic Microbial Mat Community Across Environmental Gradients in a Thermal, Acidic Stream**

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Additional investigator(s): Michael Ferris, Kathy Sheehan

Objective: Two Nymph Creek sites, defined in terms of differing light, pH and temperature, will be extensively monitored over diurnal and seasonal time periods. Contemporary analyses, including modern microscopic methods and rRNA sequencing will be used to document microbial diversity and analyze the mat's microstructure at an upstream sunny site and a downstream shaded site. Changes in macro- and microscale environmental conditions in the bulk water and through the vertical aspect of the mats will be recorded. Nucleic acid sequence-based techniques will be used to monitor population changes in situ under environmental conditions that vary in space and time.

Findings: Environmental monitors are in place to record temperature at the upstream and downstream sites (HOBO dataloggers) and to monitor PAR (photosynthetically active radiation). DNA clone libraries have been prepared seasonally and are being analyzed. Sequencing is underway on Cyanidium caldarium and other eukaryotic organisms identified from clone libraries and pure cultures.

**Project title: Physiology and Geochemical Tracing of Fes/H2S Microorganisms in Subsurface Hydrothermal Systems**

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Objective: To characterize the diversity of microorganisms associated with sulfide-containing or acidic hot springs. The initial phases of the study concentrated on evidence for pyrite-forming anaerobic bacteria. Subsequent work has concentrated on recovery, preservation, and molecular analysis of thermoacidophilic archaea.

Findings: In early June 2000, we assessed and sampled a total of 74 acidic outflows in three different areas of the park. These samples consisted of water plus sediment from pools having a temperature at or above 65º C and a pH value below about 6.0. This year we emphasized sampling in different regions of the park in order to look for differences in microbial populations. In addition, however, we also wanted to test possible changes in populations over time. As a result, we targeted two new areas this year (Crater Hills/Sulphur Mountain and Geyser Creek) and returned to one area sampled last year (Ragged Hills).

Sampling consisted of scooping sediment and spring fluid into sterile 20-mL glass vials, which were sealed and transported back to Cincinnati under ambient conditions. Two modifications of this technique were evaluated for their ability to increase yield of viable cells: exclusion of air from the vials, and buffering the pH at about 4. Neither technique produced dramatically different results from the simpler method of unbuffered, aerobic storage. This year we also took a few samples of hot, acid soil.

We recovered thermoacidophilic archaea from each of the three areas sampled. Overall, about 60 percent of the samples yielded colonies by direct plating. This resulted in more colonies than could be archived; as a result, we used various microbiological criteria to identify a smaller number of isolates representing the widest range of diversity possible. After applying these criteria, approximately 915 isolates were streaked for isolation, grown in liquid culture, and stored at -70 C for subsequent analysis.

The genetic diversity of isolates is being evaluated in comparison with isolates cultured in 1999 and with isolates recovered from other parts of the world in 2000. The analyses are proceeding rather slowly due to the large number of isolates on hand. However, qualitative molecular typing based on restriction of genomic DNA suggests low species diversity in the culturable populations at all park locations examined. Furthermore, this, or a closely related species or sub-species, dominates the culturable populations at other geothermal sites evaluated, including those outside North America. Quantitative relationships will eventually be assessed using DNA sequence comparisons of multiple genes, in a collaboration with Rachel Whitaker and her Ph.D. advisor John Taylor in the Department of Plant and Microbial Biology, UC-Berkeley.

**Project title: Molecular and Functional Ecology of Hot Spring Photosynthetic Mats**

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Objective: The project combines modern microbiological, molecular biological, and ecophysiological methods for studying the microbial ecology of photosynthetic hot spring mats. The goal is to gain a better understanding of the structure and function of the photosynthetic community in hot spring microbial mats.

Findings: Main emphasis in 2000 has been on data analysis and laboratory experiments on mat samples. A

field campaign in collaboration with Drs. Ward and Ferris during late summer focussed on obtaining further microsensor data in Mushroom Spring and Nymph Creek over a diel cycle. Studies of photosynthetic performance of various cyanobacterial isolates were conducted in the lab of Dr. Ward. Together with Dr. Nübel (Ward lab) a spectral characterization of various *Chloroflexus* type isolates as well as the spectral light distribution in mat samples was obtained. In 2001 further field measurements are planned with emphasis on dark metabolism in the mats.

**Project title: Microbial Biotransformations and Ecology**

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Additional investigator(s): Andrew Laursen, Sophia Dore

Objective: To isolate microorganisms with unique metabolic activities allowing for the transformation of C-1 compounds, polycyclic aromatic compounds, and related products from the petroleum industry.

Findings: Samples obtained from thermal features in Yellowstone National Park during August 2000 have been used as innocula for enrichment cultures. Methane and methanol were provided as sole carbon and energy sources. Cultures were maintained aerobically under mesophilic and thermophilic conditions. Isolation and characterization of microorganisms capable of growing on C-1 compounds under these conditions will continue through 2001. Microbial community structure in thermal features will be studied using soil samples that remain in a freezer repository. New enrichment cultures may be started if fresh inocula are obtained in 2001.

**Project title: Bacterial Diversity of Thermophilic Photosynthetic Bacteria**

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Objective: The main objective of this research is to discover and isolate laboratory cultures of anoxygenic (non oxygen-evolving) photosynthetic bacteria from thermal environments. Photosynthetic bacteria are model organisms for the study of basic problems in photosynthesis and thermophilic phototrophs are very desirable because of their thermostable photosynthetic machinery. The long-term goal of the research is to

probe photosynthetic diversity in hot springs of various chemistries and temperatures to determine the physiochemical limits to photosynthesis. This includes isolating and characterizing new species of photosynthetic bacteria and studying their basic biological properties including physiology, biochemistry, and phylogenetic position, in laboratory cultures. All cultures of thermophilic phototrophs from Yellowstone as well as New Zealand thermal springs have been deposited in the American Type Culture Collection (ATCC) for public access by any qualified individual. This is basic research; no commercial funding or research ties exist between this project and any for-profit organization.

Findings: Sampling was done in September 2000. However, our timing was not too good this year as two of the three days available for sampling were spent at the Old Faithful Inn because of a heavy early season snowstorm. Some sampling was done in the Mammoth Upper Terraces and in a small warm acidic hot spring along the Gibbon River near Beryl Geyser. Samples were subjected to DNA extraction and PCR amplification using a series of primers specific for proteins unique to the photosynthetic reaction center of purple anoxygenic phototrophic bacteria. This work is in progress but early results seem to confirm suspicions from enrichment culture experiments that several purple bacteria inhabit these springs. Currently, two pure cultures of purple bacteria, one from each of these two springs, has been isolated and characterized but the molecular results suggest that the diversity of purple bacteria in these springs is much greater.

**Project title: Characterization of the Microbial Rhizosphere Population of Acid and Thermotolerant Grasses Associated with Hot Springs and Microbial Diversity in Thermal Soils in YNP**

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Additional investigator(s): B. Inskeep, M. Burr, L. Botero, J. Christiansen

Objective: To study the diversity and identification of the thermophilic and acidophilic organisms associated with thermophilic plants located in YNP. We are also very interested in examining the diversity of the microbial community that thrives in select thermal soil locations.

Findings: We have obtained molecular evidence that some thermal soils (Temp. = 65°C to 85°C) apparently have diverse and complex prokaryotic communities. This study is continuing as we are developing new culturing techniques to cultivate maximal numbers of different prokaryotes from these soils. Physiological and biochemical characterization of these different isolates will then follow.

**Project title: An Analysis of Soil Microbial Community Structure in an Evolving Thermal Soil Environment**

Principal investigator: Dr. Timothy McDermott
Contact info: see above

Additional investigator(s): Tracy Norris, Lina Botero, Jon Wraith, Mohamad Etayebi

Objective: The objective of this work is to use molecular methods to analyze soil microbial community succession in response to changes in soil temperature. Investigations of the biology of hydrothermal systems have added greatly to our understanding of microbial species diversity and their evolutionary relationships. However, previous studies have generally been limited to thermal systems that are well established on the time scale of human observation. The death of lodgepole pines in this study site are indicative of a very recent expansion of the underlying geothermal plumbing. In some places temperatures as high as 80 °C were recorded, which only six months previously were closer to 25 °C. This study site provides us with a unique opportunity to observe changes in microbial community structure as they are occur. This work will allow us to address questions concerning the forces affecting microbial community structure, diversity and the colonization of geothermal features by thermophilic microorganisms.

Findings: A research plot was designated and thermocouple probes were inserted in the ground at specific locations within the plot to measure soil temperature at regular intervals. Temperature data collection was initiated in November 1999. Results indicate that the research plot includes an area of expanding geothermal activity. Soil samples were collected at several sites within the research area. Extraction of nucleic acids (DNA and RNA) from these samples by conventional protocols was analyzed via PCR and denaturant gradient gel electrophoresis. Clone libraries were also constructed and are being sequenced to compare the bacterial diversity of the sampling sites. Results suggest that temperature has been a selective force in thermally impacted soils as evidenced by apparent reduced species diversity from these sites. Total viable thermophiles (isolated on a variety of medi) were significantly higher in the thermally-impacted soils relative to unimpacted soils.

**Project title: Phylogenetic Analysis of High-Temperature Ecosystems**

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Additional investigator(s): John Spear, Jeff Walker, J. Kirk Harris, Scott Dawson

Objective: Ongoing research in the park continues to focus on the survey of microorganisms in microbial ecosystems with varying solution chemistries in Yellowstone. A molecular approach based on cloning and sequence analysis of the small sub-unit (SSU, 16S RNA (rRNA)) ribosomal gene is used to determine the

microbial composition of these ecosystems. Ongoing studies include analyses of sub-aqueous and sub-aerial systems for bacterial, archaeal, and eucaryal life.

Findings: Survey of eucaryotes in anaerobic, low temperature, and high temperature environments: the anaerobic eucaryotes so far encountered display broad diversity, in the range of new genera to kingdoms. Molecular environmental studies of the microbial constituents of the eucaryal domain of life, which includes plants and animals, has never been done at Yellowstone. We have discovered novel bacterial phylogenetic divisions through molecular biological analysis from samples obtained in the park.

**Project title: Exosporial Membrane Characteristics of Thermophilic *Clostridia***

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Additional investigator(s): Prof. George Tortora, Dr. John B. Warren

Objective: To: 1) examine indigenous spore forming bacterial species from out-flow channels of neutral pH hot springs preferably containing calcium and magnesium (travertine type springs) ranging in temperature from 35 to 60 degrees centigrade; 2) examine culturable endospores from these species in terms of their growth characteristics; exosporial membrane morphology at dormancy, activation, throughout germination and at bacterial vegetative cell outgrowth; whether these endospores are capable of attachment to substrates or each other via cellular processes or exopolymer production; histochemically and biochemically, what materials enable these endospores to attach to substrates, other bacteria and form mats or floating islands in the absence of mature vegetative cells; the speed and stages of attachment for the spores capable of growing under laboratory culturing conditions; and the visco-elastic properties of the attachment using Atomic Force Microscopy; 3) document by electron microscopy (TEM and field emission SEM) the ultrastructure of the hot spring endospores from dormancy through germination and vegetative cell outgrowth; and 4) compare the attachment mechanisms of the endospores in the hot springs with the attachment mechanisms we have been studying in pathological *Bacillus* and *Clostridial* isolates.

Findings: We have isolated and have inculture 11-14 bacterial types isolated from Terrace Spring outflow channel; and 3 or 4 new bacterial varieties isolated from the Firehole cascades. Although each organism isolated thus far has individual characteristics (i.e. colony type, anaerobic vs. aerobic habitat, odor, color, staining characteristics, spore morphology etc.), we recently analyzed each isolate using the Vitek automated identification system, and predominantly found 4-5 isolates to be Bacillus licheniformis. In Firehole cascades, there seem to be multiple strains of Bacillus pumilus, as well as B. licheniformis. Initial studies seem to suggest that a number of strains of the same organisms seem to exist in these hot springs, but the spores from each strain have very different attachment capabilities, morphologies and exosporial membrane ultrastructure. Some endospores actually have intra-exosporial vesicles that are released at outgrowth, thereby anchoring the small immature vegetative cells to neighboring debris,and preventing them from

being swept away.

The Firehole cascades produced very different endospores, many of which could not be stained with traditional spore stains. When these unstaining spores were treated with carbol fuchsin, the spores stained deep red to bright pink, suggesting the presence of surface lipid. Ultrastructural studies showed that these spores had a lipid-like outer layer during dormancy, which peeled away during germination to reveal an underlying reactive exosporium with numerous attachment projections. Therefore, we have found that the unstainable (clear) endospores that we often observed in the higher temperature hot springs seem to have a covering lipid layer but can be stained with carbol fuchsin. Later during germination, the lipid layer either diminishes or is sequentially removed, which allows the spore to progressively stain more intensely with traditional stains.

We were very surprised to find that when all of the mechanisms of endospore attachment of these hot spring species were compared to the attachment mechanisms of the human pathogens we study, the hot spring endospores had numerous unique strategies for attaching themselves, as well as ways to make a supportive network to keep spores and newly released bacterial cells attached to one another or to a substrate. All of the hot spring endospores had the exosporial attachment structures seen in our pathogenic organisms (*C.sporogenes, C.difficile, C.clostridioforme* etc.). However, the hot spring organisms had additional spore anchoring systems that seem to be unique to these environments. In some cases, the hot spring spores made a "spider web" type network interconnecting individual spores. But in other cases the material holding the endospores together was very mucoid, and in one isolate a very heavy, ruthenium red-osmium exopolymer securely tethered the spores to other spores or a substrate. In colony cultures many of the mature vegetative cells provided the mucoid, and filamentous network to keep spores attached to the colony. However the spores alone (in the absence of living vegetative cells) could make adequate attachment structures to facilitate adherence and insure survival.

**Project title: Ecology of Phototrophs in Extreme Environments: Thermal and High Iron**

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Additional investigator(s): Victor Scapa, Niki Parenteau

Objective: We are trying to determine the distribution and role of both oxygenic and anoxygenic phototrophs in extreme environments. We are also trying to determine how these bacteria interact with each other, with other microbes, and with the physical/chemical environment. Ultimately we hope to better understand the early evolution of phototrophs and their impact on the Precambrian Earth.

Findings: This year we studied the highest temperature phototrophic microbial mats in high iron springs at Chocolate Pots. These particular mats are found around 50 degrees C. They are composed of the oxygenic *Synechococcus* and the anoxygenic *Chloroflexus*. Both of these phototrophs appear to play an important role

in this high iron hot spring. Microelectrodes were used to determine the oxygen and pH levels within the mats under light and dark conditions. The *Synechococcus* clearly produced oxygen in the light but the levels of oxygen produced in these mats were much lower than in other lower temperature mats in these same springs and in other *Synechococcus* mats studied in low iron springs elsewhere. The pH was also lower than observed in other phototrophic mats. We have found some evidence for an iron stimulation of photosynthesis in the anoxygenic phototroph, *Chloroflexus*, cultured from these mats.

**Project title: Spectral Analysis of Hyperthermophile Organisms, Yellowstone National Park**

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Additional investigator(s): Jeffrey R. Johnson, Mike Ferris

Objective: The objectives of the project are to determine whether hyperthermophiles have characteristic reflectance spectra which can be used to uniquely identify species. If such unique spectral characterization can be established, the next question is whether it persists after death and possible incorporation into sinter materials and for how long it might persist. To achieve this objective we acquire visible-near infrared spectral reflectance data at wavelength of 350 to 2500 nm of various hyperthermophile organisms growing under various temperatures as well as associated siliceous and carbonate sinter deposits. Targets include various living organisms, organisms that have died but remain in situ, and organic remains that have become entombed into sinter as well as pristine siliceous and carbonate sinter.

Findings: To date, reflectance data have been collected at Lemonade Creek, Nymph Creek, Octopus Spring, Mushroom Spring and in the Upper Geyser Basin. From analysis completed, it can be concluded that the hyperthermophilic organisms do have characteristic spectra. Reflectance data for Thermocrinis ruber, *Synechococcus* lividus, *Chloroflexus* aurantiacus , Mastigocladus laminosus and Cyanidium caldarium from Octopus and Mushroom Springs and Nymph Creek show that each has a characteristic spectra significantly different from each other. Reflectance data for siliceous sinter from Mushroom and Octopus Springs exhibits absorptions which can be attributed to organic materials incorporated into the sinter. Comparison with sinter from the Upper Geyser Basin, which lacks macroscopic hyperthermophile populations, shows that ordinary sinter does not exhibit such organic signatures.

**Project title: Isolation and Characterization of Thermophilic Microorganisms**

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Objective: Isolation and characterization of thermophilic microorganisms, and the purification and characterization of their enzymes.

Findings: Microbial fieldwork included two brief visits to Yellowstone in 2000 to sample a lower- temperature hot spring where we had previously obtained high levels of thermophilic *Naegleria* isolates and where there has been an increasing level of recreational hot potting (e.g., Huckleberry Hot Spring and the springs along Polecat Creek near Flagg Ranch).

We also participated at the Seventh Interagency Science Conference to present the results of our earlier *Naegleria* survey to encourage other Yellowstone researchers to consider this area of study of eukaryotic microorganisms in the Yellowstone Ecosystem. We obtained some further samples of previously studied sites (White Creek, etc.) in an ongoing study of the types and populations of the thermophilic microorganisms present in these sites. Results include: 1) The previous survey for thermophilic *Naegleria* in the Greater Yellowstone Ecosystem (presented at the 1995 Yellowstone Conference) has been published and other investigators are reporting *Naegleria*-like 16S rRNA sequences in their Yellowstone environmental samples. One can anticipate that this will be a productive new area of investigation in Yellowstone. 2) Samples from Mushroom hot spring have been shown to have significant levels of *Thermus (Meiothermus) ruber.* Previous sampling had shown that these gram negative, red pigmented bacteria, which have an optimum growth at 60 C, are often present in many of the lower temperature hot springs in the Yellowstone ecosystem (e.g., the springs upstream in the White Creek area and at Huckleberry/Polecat Hot Springs) and the lower portions of runoff channels of higher temperature hot springs. 3) Yellow, spore forming bacterial isolates obtained from Octopus, Twin Butte Overlook, and Calcite Hot Springs and postulated to be *Bacillus flavothermus* have been confirmed to be *Bacillus flavothermus* as determined from the nucleotide sequence of their16S rRNA. *Bacillus flavothermus* differs from *Bacillus stearothermophilus* and higher temperature Yellowstone isolates (e.g., *Bacillus caldolyticus*) by its ability to grow both at 55-60 and at 25-37 C. The rather broad range of growth temperature shown by *Bacillus flavothermus* may play some role in its rather widespread distribution. These isolates are also consistent with the 16S rRNA sequences and isolate obtained from the runoff channel from Octopus Spring by Dr. David Ward and his research group at Montana State University. 4) Another apparently new isolate is a member of the *Exiguobacterium* genus that has been obtained from lower temperature hot springs (30-35 C) in Yellowstone and western Colorado. These are gram positive, low G+C, non spore-forming bacteria and determination of their 16S rRNA sequence indicates that they are distinct from bacteria previously found in thermal areas.

**Project title: Geochemical Constraints on the Ecology of the Deep Lineages within the Bacteria and Archaea**

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Additional investigator(s): Dr. Everett Shock, Dr. Cristina Takacs

Objective: To 1) determine the microbial diversity and geochemistry associated with high temperature thermal springs in YNP and 2) study the ecology of microbial communities inhabiting YNP thermal springs.

Findings: Our research in 2000 was focused on Calcite Springs and Obsidian Pool. We collected extensive geochemical and molecular biological samples along chemical and physical gradients in the springs. Additionally, enrichment culture techniques were used to isolate novel thermophilic microorganisms. Initial results indicate that the geochemistry and community structure of the springs is dynamic on a spatial and temporal scale. Our research in 2001 will focus on linking geochemical and community differences and using our cultures to understand the physiological diversity of Calcite Springs and Obsidian Pool.

**Project title: Analysis of Metal Resistance in Yellowstone Bacteria**

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Additional investigator(s): Dr. Barrie Johnson

Objective: Identify and characterize heavy metal resistant bacteria from thermal features within YNP.

Findings: This year's sampling focused again on the Norris area. Samples were obtained north of the main Norris geyser basin (Frying Pan Hot Spring), and south of the main Norris area near Tantalus Spring. Enrichments were performed to recover a variety of acidophilic bacteria found in these thermal features. In the laboratory, we have also supported Montana State University's Thermal Biology Institute in their analysis of thermophilic viruses, through DNA sequencing of clones they have generated.

**Project title: Characterizing DNA Methylase and Restriction Enzyme Genes in Environmental DNA**

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Additional investigator(s): David Ward

Objective: To discover novel restriction enzyme and methylase genes in environmental DNA samples.

Findings: We collected samples in three areas:

1. White Creek (Mushroom Spring - 22 samples; Octopus Spring - 29 samples; Pine Spring - 17 samples; and several other unnamed springs along White Creek for which we made up our own names such as "Bath Tub" or "Black Squid" - 23 samples from these sources). Total samples: 91, total consumed to date: 40, (the rest are stored at -70). We collected samples of biomass (filaments or mat) from 0.5 ml to 15 ml, with most samples being 0.5, 1.5 or 3.6 ml, and also samples of sediment (mostly 15 ml or 50 ml, since these will have lower titer of organisms). Some samples are duplicates of the same material to be used for different DNA extraction techniques (i.e., Mo Bio samples are placed directly into the manufacturer's tube containing buffer and beads for bead beating). Thus, the total number of samples is higher than the number of unique environments sampled. Samples consumed: All the "Mo Bio" samples have been consumed to make DNA (using the bead beating method). This includes 13 of the samples from Mushroom Spring, 11 samples from Octopus Spring, 7 samples from Pine Spring, and 3 unnamed samples. In addition, 3 other Mushroom samples and 3 other Octopus samples have been converted to DNA.

2. Nymph Creek. Sites at several sources along the creek and three small pools (no formal names for these). 31 samples collected (some are duplicates for different DNA extraction techniques (ie, Mo Bio samples are placed directly into the manufacturer's tube containing buffer and beads for bead beating). We collected samples of biomass (filaments or mat) from 0.5 ml to 15 ml, with most samples being 0.5, 1.5 or 3.6 ml, and also samples of sediment (mostly 15 ml or 50 ml, since these will have lower titer of organisms). Consumed: 11 samples have been consumed to make DNA (the Mo Bio ones).

3. Mammoth Springs Upper Terrace area. 52 total samples collected: 22 from Tangerine Spring and 30 others from various sources. 25 samples have been consumed to make DNA (the ones labeled "Mo Bio").

**Project title: Genetic Analysis of Brucella from Bison and the Generation of a PCR-Based Diagnostic System for Epidemiological and Ecological Studies**

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Additional investigator(s): Dr. Regina Redman, Dr. Frank Roberto

Objective: The objectives of this work are to 1) determine the genetic complexity of *Brucella* isolates from a variety of animal hosts; 2) develop a high sensitivity PCR based diagnostic system to identify the presence of *Brucella* isolates; 3) develop a PCR based diagnostic system to track specific genotypes of the

*Brucella* isolates and 4) develop a PCR based diagnostic system to discriminate live *Brucella* cells from dead cells. In addition, studies will be performed to convert the diagnostic systems to field adaptable systems capable of simple and rapid data generation.

Findings: We have completed the genetic analysis of *Brucella* isolates from several animal hosts including bison, cattle, and elk. These data are currently being incorporated into a scientific manuscript. In addition, several PCR primer sets have been prepared that amplify products specifically from *Brucella abortus* isolates. Protocols have been developed for extracting *Brucella* cells from blood samples and detection using the PCR diagnostic system. This year, genotype-specific PCR primer sets will be generated for tracking isolates in the field and studies will begin to establish a diagnostic system to differentiate live and dead cells.

**Project title: Microbial Physiology and Ecology: DNA Damage and Photosynthesis**

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Objective: The objective of this project is to study diurnal patterns of organismal physiology (e.g., photosynthesis, DNA synthesis) in order to better understand evolution on early earth and the way organisms function in their environment today. In 1999, the focus was on the effect of two naturally-occurring DNA damaging agents on DNA synthesis rates, ultraviolet radiation and hydrogen peroxide.

Findings: In 2000, research focused on the effects of UV radiation and hydrogen peroxide on microbial mat communities in Norris Geyser Basin and Octopus Spring. We found that UV radiation enhances DNA synthesis rates during the day, which we interpret as being indicative of excision repair. However, previous work suggests that the damage may be due to UVA effects mediated through oxidative damage rather than the direct effect of UVB. Experiments adding hydrogen peroxide to sample showed an increase in DNA synthesis in response to small amounts of additional hydrogen peroxide, and a decrease in response to high levels, with another increase at even higher levels, about 1 mM for Octopus and *Zygogonium* mat. At the very highest concentrations of H2O2, DNA synthesis, of course, drops to zero, probably an indication of cell death. For all the mats studied, DNA synthesis stopped by 1 M H2O2. Prechallenging *Zygogonium* with H2O2 prior to measuring the effect of H2O2 on DNA synthesis decreased the subsequent rate of DNA synthesis. This is suggestive of an induction of catalase. Techniques for studying levels of catalase and superoxide dismutase were begun in collaboration with Vanessa Lancaster and Bob Blankenship, Arizona State University. These studies will be repeated and extended in 2001.

The effect of several drugs were tested on the effect of H2O2 on DNA synthesis. Caffeine (1 mM) increased DNA synthesis in the presence and absence of additional H2O2 in *Cyanidium, Zygogonium*. *Zygogonium* mats that were placed under UV opaque screens from September to June 26, 2000 showed a down regulation in DNA synthesis when finally exposed to solar radiation in contrast to mat that was left under the UV opaque screen.

**Project title: Isolation, Identification, and Characterization of Microorganisms Living in Extreme Environments**

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Objective: 1) To train and inspire undergraduate students in the field of microbiology. 2) To identify and characterize the many unidentified and novel microorganisms associated with the thermal features in Yellowstone National Park.

Findings: With monies received from the Ledford Foundation through the Appalachian College Association, I was able to bring 9 students with me to the park this past summer to learn how to do field sampling of the thermal springs. The students were able to observe and learn something about sampling techniques and laboratory techniques in molecular biology. All of the samples I obtained from the park were consumed and I will return again in the summer of 2001 to obtain more samples.

**Project title: Diversity and Habitat Range of Sulfate-Reducing Microorganisms**

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Additional investigator(s): Sue Fishbain, Jesse Dillon, Heidi Gough, Amy Dahl

Objective: Our research at Yellowstone National Park has focused on better defining the diversity of sulfate-reducing bacteria along environmental gradients of pH and temperature. Organisms having the capacity to respire sulfate drive a key step in the global cycling of sulfur and are likely an important biological presence in many of the sulfur-rich geothermal areas within Yellowstone National Park. A long-term objective is to better define the environmental limits of dissimilatory sulfate reduction.

Findings: The recovery of deeply-diverging phylogenetic lineages, as defined by DSR gene sequence divergence, suggests that our current understanding of this important functional group of microorganisms is incomplete. Our combined analyses of different regions throughout the park suggest that sulfate respiration is a significant biogeochemical process in many of Yellowstone's geothermal features. Study sites include Bath Lake Vista, New Pit Spring, and Rolands Well Spring within the Mammoth Hot Springs

Region, Octopus and Mushroom Springs, 2 sites at the Nymph Creek area denoted as Nymph Creek and Black Sediment Pool, 4 sites in the Washburn region denoted as Site A, B, Acid Inkpot and Inkpot, 5 sites at Norris 100 Springs Plain denoted as Sites C, D, E, Cinder and Black Spring, and Obsidian Pool and Moose Pool in the Mud Volcano area. These sites provide a wide range of temperature and pH gradients (38 degrees-91 degrees C; pH 2-8). Studies in 1999 revealed significant rates of sulfate reduction at Site C, Obsidian Pool, Nymph Creek, and Black Sediment Pool. In 2000 we refined our activity assessments at these site by amending site material with alternative electron donors (CO, lactate, acetate, hydrogen, methane), testing for substrate-specific stimulation of sulfate reduction. We observed little or no stimulation at most sites, with the exception of some stimulation by hydrogen. In order to further constrain the contribution of sulfate respiration to total microbial production we determined the total phospholipid content of sediments collected from the different study sites.

**Project title: Isolation of New Hyperthermophiles and Investigations of Hyperthermophilic Biotopes**

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Objective: Isolation of new hyperthermophiles and investigations of hyperthermophilic biotopes.

Findings: During a field trip in Yellowstone, samples from different hot springs (e.g. Nymph Lake area, Ojo Caliente, Norris Geyser Basin) were taken for microbiological investigations.

**Project title: Integrated Biogeochemical Database**

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Objective: The objective of the project is to develop an Internet-accessible database that integrates microbial diversity data with geochemical information and geographical location.

Findings: We report the development of a prototype Internet accessible database and Geographic Information System (GIS) application (http://remus.inel.gov/ynphome).

**Project title: Development of Harsh Environment Biosensors**

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Additional investigator(s): William Apel, Diane Key, Kastli Schaller

Objective: The objective of this study is to culture thermophilic microorganisms from Yellowstone hot springs. These organisms will then be tested for the presence of various enzymatic activities and the enzymes will be isolated and purified from organisms showing high levels of activity. The enzymes will be characterized to determine how high temperatures affect their activity and stability compared to low temperatures versions of these enzymes. After the enzymes are characterized, they will be utilized for development of biological sensors.

Findings: On March 29, 2000, water and water/sediment samples were taken from the Norris Geyser Basin area. These samples were used as inoculum in defined media for bacterial enrichments. The sample identification is listed below, along with the location, temperature, and pH of each sample: BSno.1: Beryl Spring, 85.1, 7.08; BSno.2, Beryl Spring, 79.2, 7.20; BSno.3, Beryl Spring, 59.9, 2.21; CSno.1, Cistern Spring, 82.8, 5.64; CSno.2, Cistern Spring; VGno.1, Veteran Geyser, 58.5, 7.52; NNno.1, Pool below Veteran, 69.0, 4.16; NNno.2, Pool after Pearl Geyser, 82.1, 3.37; YFSno.1, Yellow Funnel Spring, 87.2, 3.87; GDSno.1, Green Dragon Spring; CRSno.1, Crater Spring, 74.1, 4.39; EGno.1, Echinus Geyser, 62.4, 6.73; SDno.1, Steamboat, 76.2, 6.75. The samples were stored in an 80°C portable Igloo heater for transportation to the lab. Temperature and pH were not taken for CSno.2 and GDS no.1 due to the sample location.

Upon returning to the laboratory, the samples were immediately placed into defined liquid or semi-solid media and incubated at either 70°C or 60°C on tabletop shakers. Three media were chosen: 1) Sulfur medium (Handbook of Microbiological Media), pH 2.5; 2) Sulfur medium, pH 4.8, or; 3) Thermus medium (ATCC no.697), pH 7.5. The Sulfur medium was modified to contain 0, 10, 100, or 1000 ppm arsenate and the Thermus medium was modified to contain either 0 or 10 ppm arsenate. After 4 days of incubation, growth was visually observed in thermus media containing 10 ppm arsenate inoculated with the following samples: VGno.1, EGno.1, BSno.2, SDno.1, NNno.2, CSno.2, and BSno.1. After 12 days of incubation, growth was observed in NNno.2 which was inoculated into sulfur media pH 2.5 1000 ppm arsenate. Growth was also observed in SDno.1, VGno.1, BSno.2, and EGno.1 inoculated into sulfur media pH 4.8 at 10 ppm arsenate. Growth was observed in YFSno.1 in 1000 ppm arsenate sulfur media and 100 ppm arsenate sulfur media. By day 21, growth was observed in the following enrichments and each were transferred into fresh medium: EGno.1, BSno.2, VGno.1, SDno.1, BSno.1 and CSno.2 (all in Thermus 10 ppm arsenate media), as well as NNno.2 in sulfur media pH 2.5 0, 10, 100 and 1000 ppm

arsenate and BSno.3 in sulfur media pH 2.5 100 ppm arsenate. Transfers into fresh medium continued every couple of weeks there after. No growth was ever observed with samples plated onto semi-solid media.

**Project title: Ecology of Hot Spring Microbial Communities**

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Objective: The general objective of our research is to understand the distribution and activity of microorganisms inhabiting microbial mat communities in geothermal effluents. At the moment, we are particularly interested in understanding the composition, structure and physiology of these mat communities, as models of microbial communities in general. We are using ribosomal RNA (rRNA), intervening transcribed spacer (ITS) and lipid biochemical cell components to identify community members. Our work relates to evolutionary microbiology in the sense that these gene sequences give phylogenetic information, and the association of lipids with their microbial sources helps us interpret the chemical fossil record produced by organic geochemists. In addition, we are attempting to evaluate whether the stable carbon isotope ratios of specific lipid biomarkers might help distinguish modern mat communities constructed by either cyanobacteria or green nonsulfur bacteria and hence their stromatolite counterparts in the fossil record.

Findings: During 2000 we made the following major observations: *Cyanobacteria, Synechococcus* populations in Mushroom Spring show a similar temperature distribution to those in Octopus Spring. We are currently examining their vertical distribution at four temperature-defined sites where we characterized light and chemical parameters using microsensors (Kühl). We succeeded in cultivating tens of *Synechococcus* isolates and are in the process of characterizing them genotypcially and phenotypcially with respect to possible adaptations to light and temperature. We are in the process of completing a study that suggests that genetically unique *Synechococcus* occur in hot springs around the world and even within Yellowstone. Green nonsulfur bacteria. We are completing a study of 16S rRNA-based diversity that suggests a remarkable diversity of such organisms in Mushroom Spring. We have continued experiments to evaluate the autotrophic metabolism of green nonsulfur bacteria by (1) using microsensors (Kühl) to demonstrate that the potential electron donors hydrogen and sulfide occur in the photic zone of mats containing cyanobacteria and green nonsulfur bacteria (i.e., Mushroom Spring and Tangerine Spring) in the morning and evening, and (2) conducting further 13C labeling studies at such times of day. We have extended our geochemical studies to investigate the 13C content of sugars and polyhydroxy alkanoic acids, which lead us to a competing hypothesis that heavy isotopic signatures of green nonsulfur-like bacteria in the mats may relate to the unique physiological situation involving major carbon flux through polysaccharides.

**Project title: Isolation and Characterization of Thermophilic viruses from Yellowstone National Park**

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Objective: To isolate and characterize thermophilic viruses from the thermal features of Yellowstone National Park.

Findings: Six different particle morphologies were found in *Sulfolobus* enrichment cultures grown at 80C and pH 3. Virus and virus-like particles were readily detected in a high proportion of enrichment cultures (43 percent), suggesting that viruses are a common feature of *Sulfolobus* species in YNP. Three of the particle morphologies are similar to viruses previously isolated from *Sulfolobus* species from Iceland and/or Japan. Three virus particle morphologies have not been previously observed from thermal environments. Some of these morphologies appear to be completely novel.

Despite the fact that the YNP SSV-like, SIRV-like, and SIFV-like particles have nearly identical morphologies to *Fusellovirus* (SSVs), *Rudiviruses* (SIRVs) and *Lipothrixviruses* (SIFV) isolated from Japan and Iceland, limited analysis of their genomic sequence indicates that they are only distantly related. This genomic diversity may reflect their long-term geographic isolation or it may be a function of adaptation to unique features of *Sulfolobus* species present in YNP. Ongoing studies are aimed at addressing the level of diversity within YNP as compared to related viruses present in other thermal regions of the world.

In contrast to the diversity of virus types, there does not appear to be a similar host diversity. The limited analysis of the 16S rDNA from several of the YNP enrichment cultures that were sequenced indicates that the Sulfulobus host species are nearly identical. The apparent contradiction of low host diversity with high virus diversity is intriguing. A thorough analysis of viruses present in *Sulfolobus* species is likely to provide unique insights into biochemical adaptations to life in extreme thermal environments. In addition, a detailed understanding of the viral replication cycle in *Sulfolobus* species will likely provide insight into cellular process present in Archaea and lead to a more thorough understanding of this unique domain of life.